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Blood Vessel Fibronectin Increases in Conjunction with Endothelial Cell Proliferation and Capillary Ingrowth During Wound Healing

RICHARD A. F. CLARK, M.D.,* PATRICIA DELLAPELLE, B.S., ELEANOR MANSEAU, B.S.
JOAN M. LANIGAN, B.S., HAROLD F. DVORAK, M.D., AND ROBERT B. COLVIN, M.D.

Departments of Dermatology and Pathology, Massachusetts General Hospital, Beth Israel Hospital, and Harvard Medical School, Boston, Massachusetts, U.S.A.

The regulation of angiogenesis and alterations in the structure of blood vessels taking part in wound healing are poorly understood. In studies of guinea pig 4-mm skin wounds, left uncovered for 1–28 days, biopsied and processed for 1-μm section and immunofluorescence, we found that fibronectin in blood vessel walls markedly increased in conjunction with endothelial cell proliferation and capillary ingrowth. Both the endothelial cell proliferation and the increased vessel wall fibronectin were restricted to a 0.5-mm area along the margin of the wound and occurred 3–7 days after injury. Fibronectin was easily demonstrated in capillaries of the peripheral granulation tissue but was difficult to demonstrate in central areas of the granulation tissue secondary to a brightly fluorescent reticular background staining probably attributable to fibroblast-related fibronectin. The fibronectin in blood vessel walls rapidly diminished as endothelial cell proliferation and capillary ingrowth ceased. These data suggest that fibronectin may provide a provisional substratum for endothelial cell mitosis and movement.

In the process of embryogenesis and wound healing, a specialized interaction must exist between mobile cell populations and their substratum to provide a sufficient degree of adherence to ensure attachment while permitting cell movement and mitosis. The ideal situation might be for the cells to control the degree to which they adhere to their substratum. Recent *in vitro* investigations have examined factors responsible for variations in cell adhesion to underlying substratum. Many cells bind to tissue culture substratum through extracellular glycoproteins [1–15]. One of these attachment proteins, fibronectin, is produced by fibroblasts [16–18], monocytes [19,20], and endothelial cells [21–23], as well as other cell types and is present at moderate concentrations in blood and serum [24]. In culture, fibronectin, derived from serum and/or produced by cells, can bind to the substratum and simultaneously bind to cell surfaces [6,13,14]. In doing so, fibronectin attaches cultured cells to the underlying surface. Culp and his coworkers [25,26] recently have postulated that fibroblasts may modulate their binding to fibronectin by locally modifying the concentration and type of glycosaminoglycan on their surface membrane. If this hypothesis is correct, not only for fibroblasts but also for endothelial cells and epidermal cells, it would provide a mechanistic explanation for the appearance of fibronectin in morphogenesis dur-

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Reprint requests to: Dr. Richard A. F. Clark, Department of Medicine, National Jewish Hospital, Denver, Colorado 80206.

Abbreviations:
CIG: cold-insoluble globulin

ing fetal development [27–29], explant outgrowth in tissue culture [30], and in tissue repair [31–35]; that is, fibronectin may act as a provisional substratum which can support cell migration and localization by permitting control of adherence at the cellular level.

We have reported previously that marked increased blood vessel wall fibronectin occurred at times when endothelial cells were activated [31] and proliferating [36]. This report further extends and supports the observations that fibronectin occurs in areas of intense endothelial cell mitosis and movement [31].

MATERIALS AND METHODS

Wound Healing Model

Standard wounds were made with a 4-mm skin biopsy punch down to the panniculus carnosus in the chemically depilated flanks of 400–600 g male or female Hartley guinea pigs. At various time intervals thereafter, ranging from 1 to 28 days, the uncovered wound sites were harvested and processed for immunofluorescence and 1-μm thick Epon sections as previously described [35].

Antisera and Antibodies

Guinea pig fibronectin and fibrinogen were purified as previously described [31,37] and used as immunogens in rabbits. Antibodies were purified, tested for specificity and sensitivity, and conjugated with fluorescein for direct immunofluorescence [31].

Anticollagenous basement membrane protein (type IV collagen) antibodies were elicited with human type IV collagen and with type IV collagen purified from EHS mouse sarcoma [35,38]. The latter was kindly provided by Dr. George Martin.

Anti-von Willebrand antigen-related antibodies (anti-factor VIII) (Atlantic Antibodies Co., Scarborough, Maine) stained bovine aorta endothelial cells but not W138 fibroblasts. All fluorescence activity was depleted when the antibodies were first absorbed with factor VIII preparations (Hyland Laboratories, Inc., Glendale, California) but not depleted after absorption with von Willebrand factor-deficient plasma.

Autoradiography

In some experiments, animals were injected with 500 μCi of [^{3}H]-thymidine (60 μCi/mmol) i.v. 1 hr prior to wound harvest. One-micron

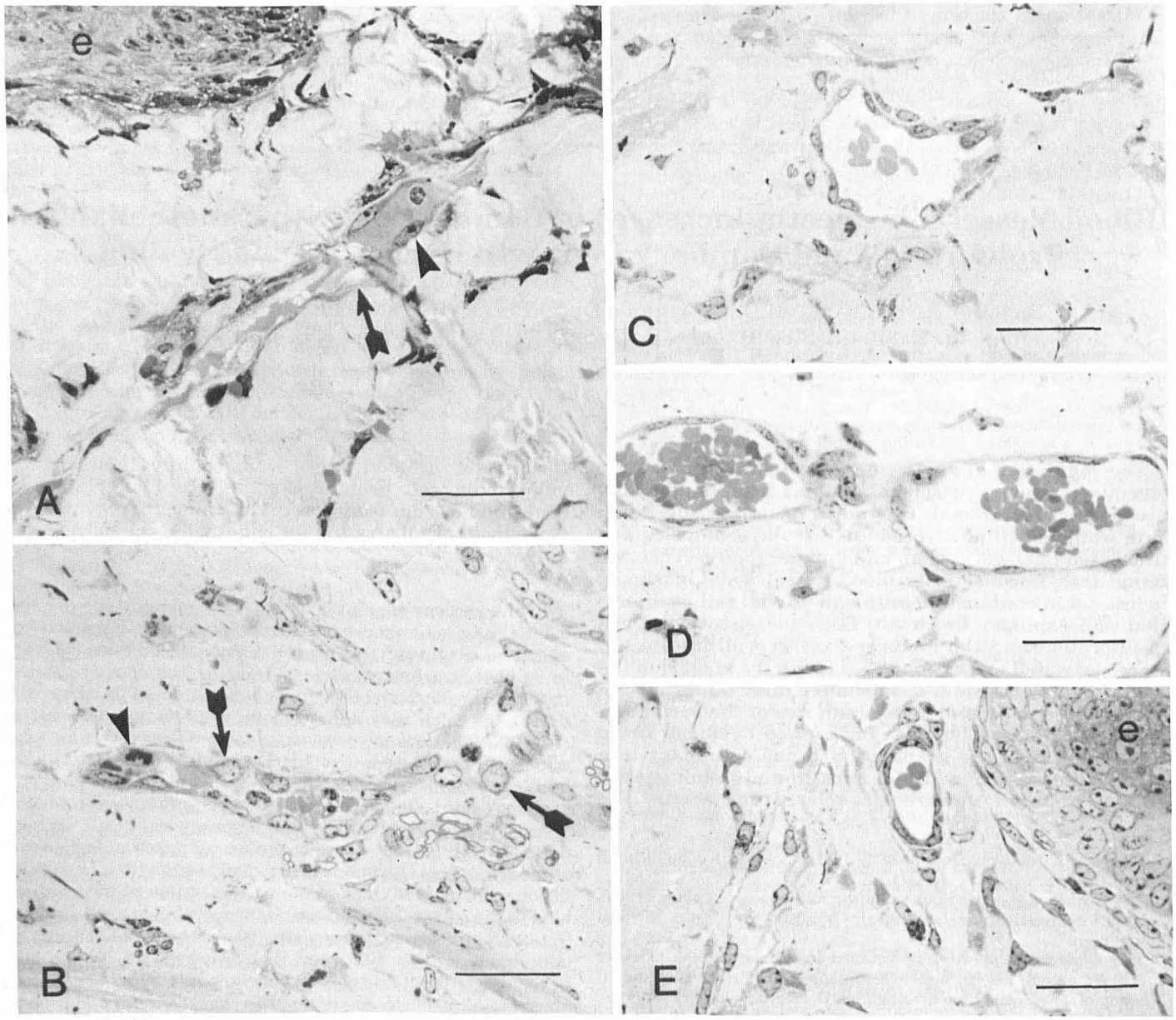

FIG 1. One-micron Giemsa-stained sections of small blood vessels within 0.5 mm of the edge of an excisional wound in a guinea pig at various times after injury. *A*, Severely damaged vessel at 2 days contains necrotic (*arrow*) and injured (*arrowhead*) endothelial cells. *B*, Vessel at 3 days has activated endothelial cells (*arrows*) and an endothelial cell undergoing mitosis (*arrowhead*). *C* and *D*, Vessels at 5 and 7 days, respectively, whose endothelial cells are no longer activated, are markedly dilated. *E*, Vessels at 12 days appear normal. Bars = 25 μm.

sections were prepared as described [35], coated with Kodak NTB-2 emulsion, and exposed for 2 weeks prior to Giemsa staining [39].

For purposes of quantitation, nuclei with more than 5 grains were considered to be labeled. The percentage of labeled endothelial cells were determined with an ocular micrometer by counting all endothelial cells within 2 swaths, each 0.5 mm in width, taken vertically along the wound edge from the epidermis to the panniculus carnosus. Similar swaths through normal skin were counted to establish baseline control values.

RESULTS

THE BLOOD VESSELS OF HEALING WOUNDS

Appearance of Blood Vessels in Healing Wounds in 1-μm Epon Sections

During the first 2 days after excisional wounding, blood vessels within 0.5 mm of the wound edge showed signs of acute injury as demonstrated by the blood vessel in Fig 1*A* which contains both a necrotic endothelial cell characterized by its smudged almost homogenized nucleus and an injured endothelial cell characterized by its dark-staining nucleus and cytoplasm. By day 3, however, injury was no longer apparent and endothelial cells appeared activated with enlarged open nuclei and expanded cytoplasm so that endothelial cells bulged into the vessel lumen. Occasional mitotic figures were seen (Fig 1*B*). Between 5 and 7 days after injury, many blood vessels near the wound edge and base appeared abnormally dilated and endothelial cells were thinner and appeared much less activated (Fig 1*C, D*). By 10–14 days, most blood vessels near the wound edge had returned to normal (Fig 1*E*).

Autoradiography of Blood Vessels Near the Wound Edge

The amount of labeling of endothelial cells with [^{3}H]-thymidine was dependent on the distance individual vessels were from the wound (Fig 2). Vessels ≤ 0.5 mm from the wound edge attained the greatest peak values (just under 50% of total endothelial cells) on day 2; these data correlate with the increased mitotic figures observed in such vessels at day 3. Thereafter, the extent of labeling fell rather precipitously over 1–2 weeks. Endothelial cells of vessels 0.5–1.0 mm from the wound edge also showed maximum labeling on day 2; however, never more than 10% of cells were labeled and thymidine incorporation returned to normal within a week. Thus, endothelial labeling was sharply confined to vessels immediately adjacent to the wound, which correlated well with morphologic observations that neither vessel injury nor mitosis occurred outside of this zone. New vessels entered the wound as part of the healing process after day 3, and endothelial cells of such vessels exhibited significant labeling over the interval of 5–9 days.

Immunofluorescence Studies of Vessels Near the Wound Edge

At 1 and 2 days after injury, vessels within 0.5 mm of the wound edge showed normal basal lamina staining with fluorescein-labeled antifibronectin antibodies (Fig 3*A*). By 3 days, however, occasional vessels near the wound edge exhibited a significant increase in staining intensity (Fig 3*B*), and at 5–7 days this change became prevalent. There was a marked and consistent increase in staining for fibronectin of vessels within 0.5 mm of the wound edge (Fig 3*C–E*). By 9 days, antifibronectin staining of these vessels had returned to normal (Fig 3*F*). Vessels > 0.5 mm from the wound perimeter showed no change from normal with regard to fibronectin staining at any interval.

The blood vessels did not stain with fluorescein-conjugated antifibrinogen antibodies at any time after wounding, suggesting that nonspecific absorption of plasma proteins could not account for the increased fibronectin staining. Moreover, prior absorption of the antifibronectin antibodies on a fibronectin affinity column eliminated the staining of these blood vessels while absorption on a fibrinogen affinity column did not.

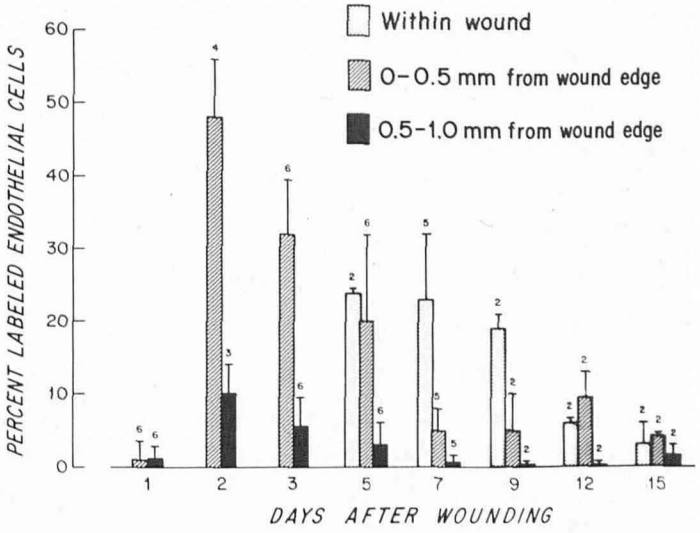

FIG 2. Depicts percentage of proliferating endothelial cells 0–0.5 mm and 0.5–1.0 mm from the wound edge and within the granulation tissue as a function of time. Each *bar, bracket,* and *number* represents the mean ± standard error of the mean of percentage thymidine-labeled endothelial cells for n number of animals for the data point, respectively.

CONNECTIVE TISSUE ELEMENTS OF WOUND HEALING

One-Micron Sections and Autoradiography of Developing Granulation Tissue

Fibroblast and capillary ingrowth began between 3–5 days after punch biopsy and first appeared at the wound base. Endothelial cells within the wound showed notable proliferation, as measured by [^{3}H]-thymidine incorporation, at day 5 after tissue extirpation and for the subsequent 4 days (Fig 2). By day 5, a 0.5–1.0 mm bed of granulation tissue had formed at the base of the wound (Fig 4*A*). The newly formed blood vessels were for the most part oriented vertically, running perpendicular to the skin surface. Fibroblasts at first had no particular orientation; however, by 7 days, the more superficial granulation tissue fibroblasts beneath the newly formed epidermis became oriented horizontally, parallel to the skin surface; blood vessels maintained their vertical orientation (Fig 4*B,C*). The contrast in fibroblast and microvascular orientation became even more pronounced at day 10 (Fig 4*D*). With continued proliferation of fibroblasts and capillaries below, the migrating or newly formed epidermis was gradually elevated to the level of the epidermis of the adjacent normal skin by 12–15 days.

Immunofluorescence Studies of Granulation Tissue

By 5 days, when granulation tissue had begun to accumulate at the base of the wound (Fig 4*A*), blood vessels in the subcutaneous tissues below the granulation tissue appeared dilated and stained brightly for fibronectin (Fig 5*A,B*). The microvasculature within the adjacent granulation tissue was also stained intensely for fibronectin (Fig 5*A–C*). Since the entire granulation tissue gave a fine reticular fluorescent pattern when stained with antifibronectin antibodies, it became increasingly difficult to distinguish blood vessels from the reticular fluorescent background staining in areas of the granulation tissue even a short distance from the wound edge (Fig 6*D*). However, blood vessels in such areas could be readily identified in 1-μm sections (Fig 6*A*) and by other fluorescent probes: anti-type IV collagen (Fig 6*B*) and anti-factor VIII (Fig 6*C*). The reticular fluorescence of the granulation tissue observed with antibodies to fibronectin was oriented parallel to the skin surface (Fig 6*D*), corresponding to the orientation of fibroblasts as described above. This fluorescent pattern was specific for fibronectin because little or no staining was observed with fluorescein-conjugated antibodies

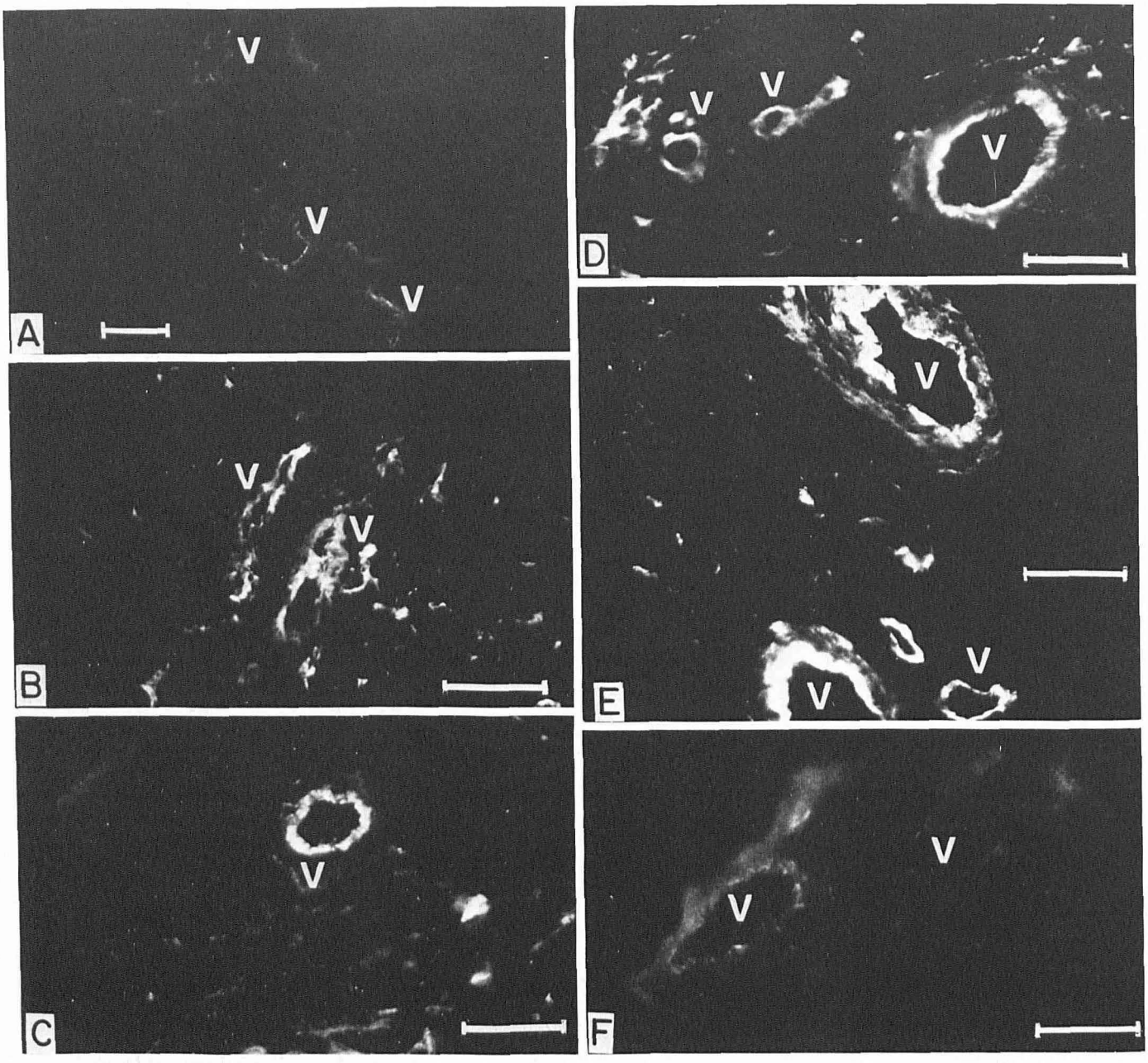

FIG 3. Immunofluorescence studies of small blood vessels (*v*) within 0.5 mm of an excisional wound in guinea pigs at various time periods after extirpation using fluorescein-conjugated anti-guinea pig fibronectin antibodies. *A*, At day 1 vessels stain weakly. *B*, Vessels at day 3 often stain brightly for fibronectin. *C–E*, From days 5–7 vessels always have intense fluorescence. *F*, By day 9 vessel fluorescence has returned to normal baseline. Bars = 50 μm.

to fibrinogen (Fig 6*E*), anti-C3, or anti-IgG. Absorption of the antifibronectin antibodies with fibronectin-Sepharose abrogated the granulation tissue fluorescence as well as the staining of blood vessels at the perimeter of the granulation tissue and in the surrounding normal dermis.

DISCUSSION

We established earlier that increased fibronectin appeared in dermal blood vessels exhibiting endothelial cell proliferation in the course of delayed-type hypersensitivity skin reactions [31,36]. These deposits were apparently not related to enhanced microvascular permeability. At 1–2 days after skin test, when reactions were maximal and dermal blood vessels exhibited abnormal permeability, increased fibronectin, presumably derived from extravasated plasma cold-insoluble globulin (CIG), was observed along with fibrin in the intervascular dermis but not in blood vessel walls. However, on day 3 after challenge, when vessel permeability had returned to normal and endothelial cell proliferation was maximal, increased fibronectin was observed in the basement membrane region of small blood vessels. Thus, a correlation was observed between fibronectin deposition in blood vessel walls and endothelial cell proliferation in these same blood vessels. This correlation was strengthened when we studied the reactions of cutaneous basophil hypersensitivity. In this form of cell-mediated hypersensitivity, neither increased fibronectin accumulation in blood vessels nor endothelial cell proliferation was observed.

The data presented here also supported a close relationship

between blood vessel fibronectin deposition and endothelial cell proliferation. In our excisional wound model, endothelial cell proliferation was almost entirely limited to a zone within 0.5 mm of the wound perimeter and occurred only between 3 and 7 days after injury (Fig. 2). Thus the increase in blood vessel wall fibronectin correlated with endothelial cell proliferation both temporally and spatially (Fig. 3*B–E*). As in delayed-hypersensitivity skin reactions, we postulate that the increased amounts of fibronectin deposited in blood vessels were produced *in situ* by activated and/or proliferating endothelial cells and were not derived from extravasation of circulating CIG.

Several groups of investigators have demonstrated that proliferating endothelial cells synthesize and secrete fibronectin *in vivo* [21–23]. Some of the secreted fibronectin is, in fact, depos-

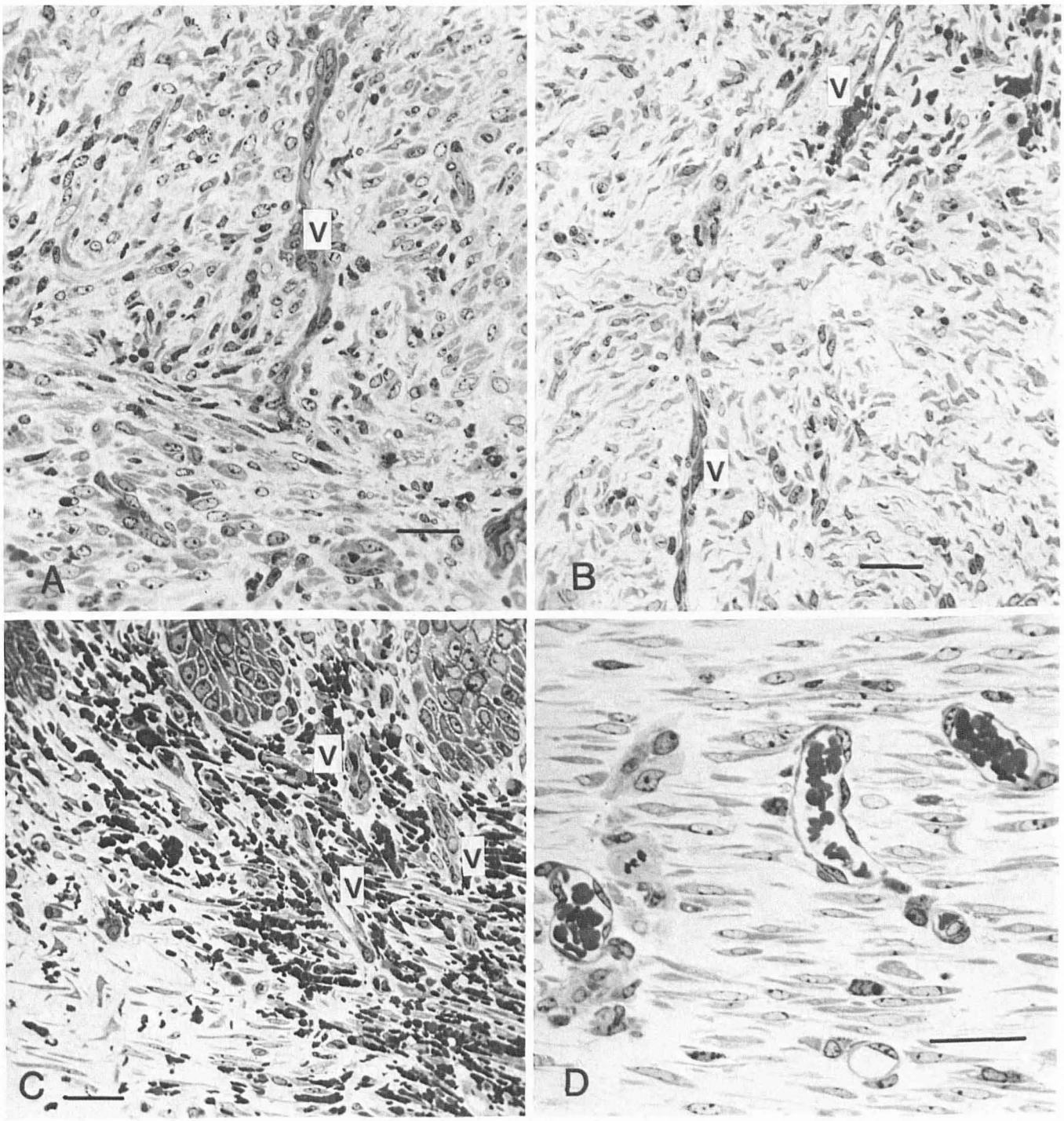

FIG 4. One-micron Giemsa-stained sections of granulation tissue in guinea pig excisional wounds. All panels have the same orientation with the epidermis (not shown in *A, B,* and *D*) lying directly above the dermis. *A* and *B,* Base of granulation tissue at 5 and 7 days, respectively, had vertically oriented blood vessels and random pattern of fibroblasts and histiocytes. *C,* Edge of granulation tissue at the epidermal interface shows again that blood vessels were perpendicular to the skin surface, but, at this location in the granulation tissue, the fibroblasts had become parallel to the epidermis. Blood vessels are indicated by *V. D,* The central granulation tissue at 10 days demonstrates even more clearly the opposite orientation of blood vessels and fibroblasts. Bars = 25 μm.

ited between the cell monolayer and the culture dish, creating a form of "basement membrane" [23]. Recently, we have presented additional evidence that proliferating blood vessels produce fibronectin *in situ* [40]. In these experiments, we made small excisional wounds in rat xenografts that had been placed on thymectomized mice treated with antilymphocyte serum to prevent immunologic rejection. Specific antisera directed against either mouse or rat fibronectin allowed us to identify the source of fibronectin deposited in vessels. In brief, only rat fibronectin was found in proliferating vessels, providing strong evidence that the increased amounts of fibronectin seen in proliferating blood vessels were indeed synthesized locally.

The data presented in this report also demonstrate that increased fibronectin occurred in the small blood vessels of the granulation tissue that filled the excisional defect (Fig 5*A–C*). Attempts to follow these small vessels within the granulation tissue were frustrated by the large amounts of the extravascular fibronectin presumably of fibroblast origin (Fig 6*D*). Thus, we cannot say with certainty whether the capillary basement membrane zone deep within the granulation tissue does [32] or does not contain increased fibronectin. In any event, the ingrowing capillary buds may depend on the presence of a provisional fibronectin matrix for their continued migration and proliferation. This possibility was supported by the observation that a marked diminution of capillary proliferation occurred within the granulation tissue 10–14 days after wounding (Fig 2) simultaneous with a marked decrease in the intensity of staining of the reticular fibronectin within the granulation tissue (data not shown). Additionally, recent evidence has been presented to suggest that fibronectin serves as a chemoattractant for endothelial cells [41].

The biologic significance of these observations has not been

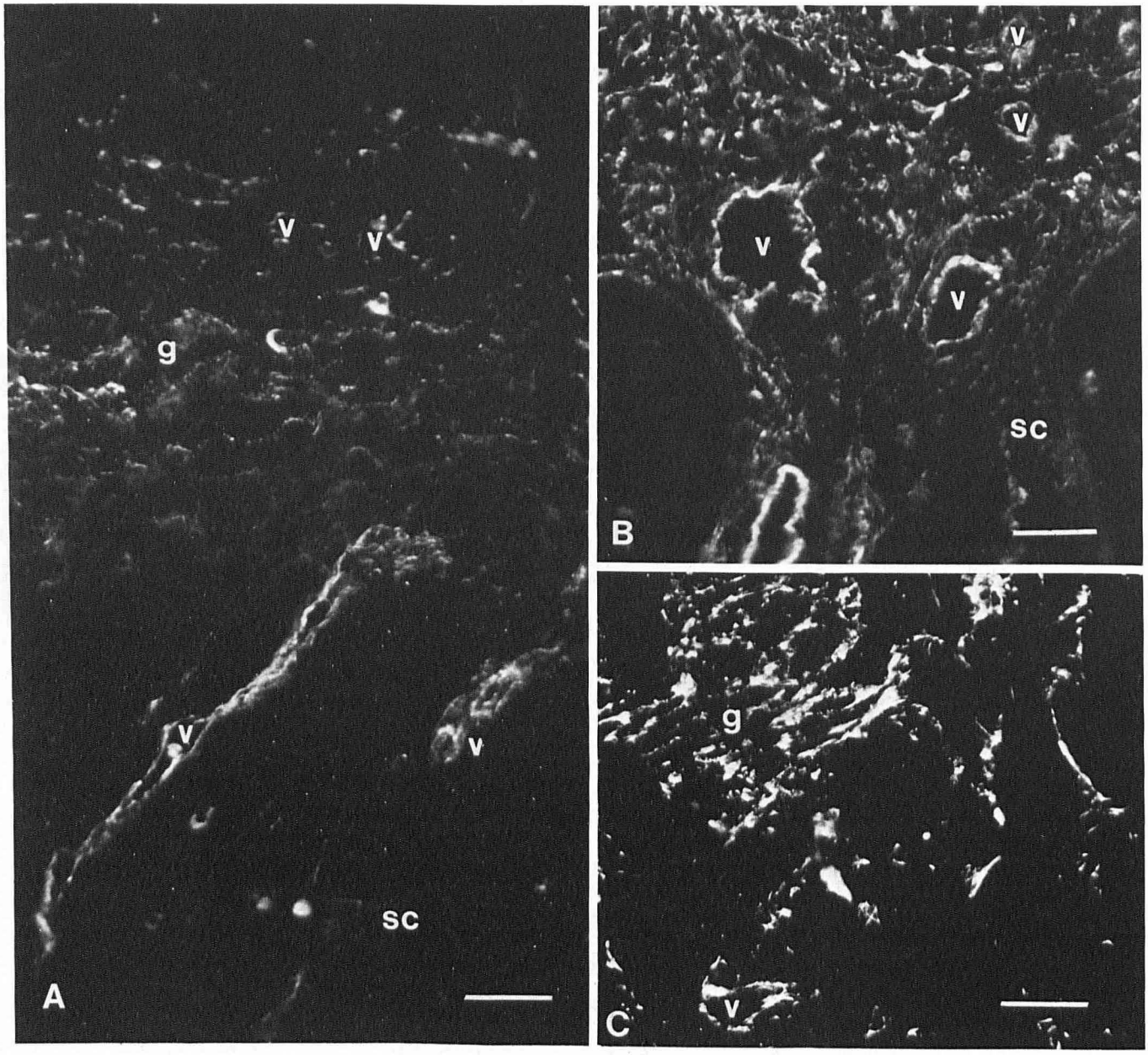

FIG 5. *A–C*, Immunofluorescence studies of the granulation tissue–panniculus or granulation tissue–dermis interface after excisional wounding of guinea pigs. Vessels (*V*) at the interface and within the granulation tissue (*g*) stain brightly with fluorescein-conjugated anti-guinea pig fibronectin. Subcutaneous (*sc*) tissue is interfaced with granulation tissue (*g*) in panels *A* and *B* while dermis is interfaced with granulation tissue (*g*) in panel *C*. *A* and *B* are 6 days and *C* is 7 days after tissue extirpation. Bars = 50 μm.

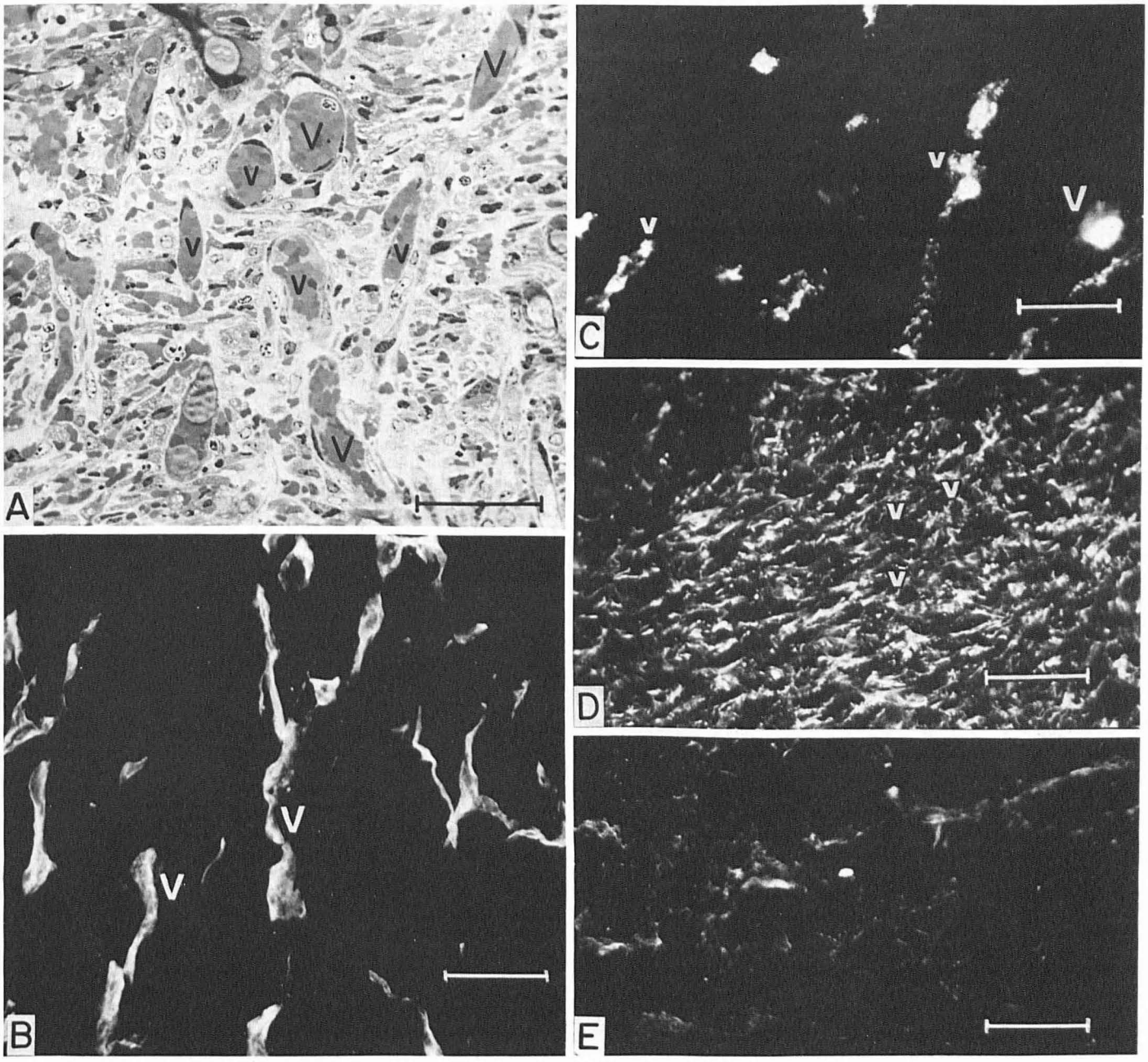

FIG 6. Immunofluorescence and 1-μm Giemsa-stained section studies of granulation tissue in guinea pigs at day 7. All panels are oriented exactly the same with the epidermis (not shown) overlying the granulation tissue. *A*, One-micron section of Giemsa-stained granulation tissue. *B*, An adjacent section stained with antihuman type IV collagen. *C*, An adjacent section stained with antihuman von Willebrand factor VIII-related antigen. *D*, An adjacent section stained with anti-guinea pig fibronectin. *E*, An adjacent section stained with anti-guinea pig fibrinogen. Bars = 50 μm.

fully elucidated; however, several potentially important possibilities may be envisioned. The increase in vessel wall fibronectin that occurs in conjunction with endothelial cell proliferation could represent a reparative process of the endothelial cell basement membrane and, additionally, could lead to modification of the structure of its abluminal surface. A transient alteration of the basement membrane might provide a more suitable matrix for endothelial movement and mitosis [12], act to reduce the coagulative nature of the subendothelium, and/or impede further inflammatory cell ingress. Further studies are needed to elucidate these possibilities.

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